Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*

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DnaA protein, the initiation factor for chromosomal DNA replication in *Escherichia coli*, is activated by ATP. ATP bound to DnaA protein is slowly hydrolyzed to ADP, but the physiological role of ATP hydrolysis is unclear. We constructed, by site-directed mutagenesis, mutated DnaA protein with lower ATPase activity, and we examined its function in *vitro* and *in vivo*. The ATPase activity of purified mutated DnaA protein (Glu204→Gln) decreased to one-third that of the wild-type DnaA protein. The mutation did not significantly affect the affinity of DnaA protein for ATP or ADP. The mutant dnaA gene showed lethality in wild-type cells but not in cells growing independently of the function of oriC. Induction of the mutated DnaA protein in wild-type cells caused an overinitiation of DNA replication. Our results lead to the thesis that the intrinsic ATPase activity of DnaA protein negatively regulates chromosomal DNA replication in *E. coli* cells. **Keywords:** ATPase/DnaA/*Escherichia coli*/negative regulation/site-directed mutagenesis

Introduction

The initiation of DNA replication has to be strictly regulated so that it is coupled with cell division. However, the molecular mechanism underlying the control of initiation of DNA replication is unclear.

In *Escherichia coli*, chromosomal DNA replication has been studied genetically and biochemically. The functions of a number of replication factors have been elucidated (Kornberg and Baker, 1992). DnaA protein plays a crucial role in the initiation of chromosomal DNA replication (Hirota et al., 1970; Kornberg and Baker, 1992). It specifically binds to DnaA boxes in the oriC region (Fuller and Kornberg, 1983; Fuller et al., 1984), and opens up the double-stranded DNA for entrance of other replication proteins (Sekimizu et al., 1987; Bramhill and Kornberg, 1988). DnaA protein has a high affinity for both ATP and ADP (Sekimizu et al., 1987). Tightly bound ATP is slowly hydrolyzed to form the ADP-binding form of DnaA protein. In an oriC DNA replication system reconstituted with purified proteins, the ATP-binding form of DnaA protein is active, whereas the ADP-binding form is inactive (Sekimizu et al., 1987). Expression of DnaAcos protein, which is not susceptible to inactivation by ADP binding, leads to overinitiation of chromosomal DNA replication in cells and in an *in vitro* oriC DNA replication system (Katayama, 1994; Katayama and Kornberg, 1994). We reported that synthesized organic compounds which were designed to block the ATP binding to DnaA protein specifically inhibited the oriC DNA replication in *vitro* (Mizushima et al., 1996c). These results suggest that the activity of DnaA protein is regulated by its adenine nucleotide binding in *vitro*.

As DnaA protein bound to ATPγS or AMPPNP, non-hydrolyzable analogs of ATP, is active in DNA replication *in vitro* (Sekimizu et al., 1987), the hydrolysis of ATP bound to DnaA may not be required for replication activity of the protein. Thus, the physiological role of the hydrolysis of ATP bound to DnaA protein remains to be elucidated. Since this hydrolysis resulted in formation of the ADP-binding form of DnaA protein, which is inactive in *oriC* DNA replication in *vitro* (Sekimizu et al., 1987), we assumed that the ATPase activity of DnaA protein would be involved in its negative regulation of replication activity. To test the above hypothesis, we constructed a mutant DnaA protein in which intrinsic ATPase activity decreases, and we examined its function in DNA replication *in vitro* and *in vivo*. For this, we constructed a mutant DnaA protein where the glutamic acid residue at position 204 was replaced by glutamine. This mutant DnaA protein maintained high affinities for ATP and ADP; however, the intrinsic ATPase activity decreased. We showed that induction of mutant DnaA protein in wild-type cells led to overinitiation of DNA replication, which means that the hydrolysis of ATP bound to DnaA protein probably negatively regulates chromosomal DNA replication.

Results

**Construction and purification of mutant DnaA protein**

It was reported that Glu203 of the F1-ATPase β subunit is required for the hydrolysis of ATP (Ohtsubo et al., 1987; Amano et al., 1994). A comparison of amino acid sequences of other ATPases revealed that this glutamic acid residue (or asparatic acid residue) is highly conserved and, hence, is probably important for the activity of the enzyme (Yoshida and Amano, 1995). SecA protein is involved in protein translocation across inner membranes of *E. coli* and has a high affinity for ATP. ATP tightly bound to SecA protein is hydrolyzed by its intrinsic ATPase activity as is DnaA protein (Cunningham et al., 1989; Akita et al., 1990; Matsuyama et al., 1990). Substitution of Asp133 of SecA protein with Asn was shown to decrease the intrinsic ATPase activity but did not affect its affinity for ATP (Sato et al., 1996). Glu204 of the DnaA protein is predicted to be a residue required for the
Characterization of ATP and ADP binding and ATPase activity of DnaA E204Q protein

We first examined the binding of DnaA E204Q protein to ATP and ADP. DnaA protein and (α-32P)ATP of various concentrations were incubated together, and the amount of ATP bound to the protein was determined by filter-binding assay (Sekimizu et al., 1987). ATP bound to both DnaA E204Q and wild-type DnaA in a dose-dependent manner. Scatchard plot analysis revealed that the $K_d$ value for the binding of ATP to DnaA E204Q was 46 nM (Figure 2A). For wild-type DnaA protein, the $K_d$ was determined to be 48 nM, much the same value as reported previously (Sekimizu et al., 1987). The numbers of ATP-binding sites per mutated and wild-type DnaA molecule were calculated to be 0.53 and 0.40, respectively. The affinity of DnaA E204Q for ATP was determined in the same manner. $K_d$ values for the binding of ADP to DnaA E204Q and wild-type DnaA were 160 and 230 nM, respectively (Figure 2B). The value for wild-type DnaA protein is twice that reported previously (Sekimizu et al., 1987). The numbers of ADP-binding sites per mutated and wild-type DnaA protein molecule were calculated to be 0.29 and 0.23, respectively. These results indicate that the mutation (Glu204 to Gln) probably does not change the affinity for ATP and ADP significantly.

Next, we compared the ATPase activity of DnaA E204Q with that of the wild-type protein. DnaA protein was pre-incubated with [α-32P]ATP at 0°C and incubated further at 38°C in the presence of DNA. The complex of DnaA protein with ATP or ADP was recovered by immunoprecipitation, using anti-DnaA serum, and adenine nucleotide bound to DnaA protein was analyzed by thin layer
Fig. 2. ATP and ADP binding to DnaA E204Q protein. DnaA E204Q and wild-type DnaA protein (1 pmol) were incubated with various concentrations of [α-32P]ATP (A) or [3H]ADP (B) for 15 min at 0°C. The amount of bound ATP or ADP was determined by filter-binding assay, as described in Materials and methods, and a Scatchard plot analysis prepared. Linear repression was performed by the least-squares methods.

Fig. 3. Time course of hydrolysis of ATP bound to DnaA E204Q protein. Hydrolysis of ATP bound to DnaA E204Q or wild-type DnaA protein was monitored on thin layer PEI-cellulose plates (A), as described in Materials and methods. The autoradiograms were scanned by densitometry and the ratio ADP/(ADP and ATP) was determined (B).

chromatography. The ATPase activity of DnaA E204Q decreased to about one-third that of wild-type protein (Figure 3), indicating that Glu204 is an important amino acid residue for the intrinsic ATPase activity of DnaA protein.

Replication activity in vitro of DnaA E204Q protein
We examined the replication activity of DnaA E204Q protein in an oriC complementation assay. As shown in Figure 4, DnaA E204Q protein was active in DNA replication. There are at least two differences between wild-type and mutated DnaA protein, with regard to DNA replication activities. One is that the specific activity (units/mg protein) of DnaA E204Q is about one-third that of the wild-type protein, the other is that the amount of synthesized DNA at a saturation level of DnaA E204Q protein is 200 pmol of nucleotides (template DNA, 600 pmol nucleotides), being about one-third that of the wild-type protein. The batch-to-batch variability in values (specific activity and DNA synthesis at a saturation level of DnaA protein) of the wild-type DnaA protein was <25%, thereby suggesting that differences in values between mutant and wild-type DnaA protein cannot be

Fig. 4. Replication activity of DnaA E204Q protein in a crude extract. DnaA E204Q (■) and wild-type DnaA protein (○) were incubated with 1 μM of ATP (▲) or ADP (●) for 15 min at 0°C. DNA replication in a crude extract was carried out as described in Materials and methods.
explained by this variability. Under our conditions, the wild-type DnaA protein purified using the present protocol showed a specific activity of 0.25×10⁸ units/mg protein (Figure 4), which is one-fifth of the previously reported value (Sekimizu et al., 1988b). The lower values are not caused by the modification of purification procedures, because wild-type DnaA protein purified according to the previously published protocol (Sekimizu et al., 1988b) also showed low specific activity (data not shown). The lower value is also not due to denaturation of DnaA protein in our preparations, since the capacity of DnaA protein for ATP binding, which is a good criterion with which to assess the denaturation of DnaA protein, was much the same (Figure 2A) as the previous published data (Sekimizu et al., 1987). We consider that the lower specific activity of wild-type DnaA protein is caused by assay conditions, because the specific activity of DnaA protein measured by mutant complementation assay varies according to the batch of the extracts and species of oriC template DNA.

Next, we examined the effect on replication activity of pre-incubation of DnaA E204Q protein with ADP. DnaA protein was pre-incubated with 1 μM of ADP, and the DNA replication reaction was performed in the oriC complementation assay system. As shown in Figure 4, the activity of DnaA E204Q protein was diminished by pre-incubation with ADP, as found in the case of the wild-type DnaA. This result suggests that the ADP-binding form of DnaA E204Q protein is inactive, like wild-type DnaA protein, i.e. the mutant protein is susceptible to inactivation by ADP binding.

We next asked whether DnaA E204Q protein is active in the oriC DNA replication system reconstituted with purified proteins. DnaA E204Q protein did show DNA replication activity in the system, in a dose-dependent manner (Figure 5). The specific activity of DnaA E204Q protein was half that of wild-type DnaA protein, and the amount of synthesized DNA at a saturation level with DnaA E204Q protein was much the same as that with the wild-type protein.

**oriC-dependent lethality by the dnaA400 gene encoding DnaA E204Q protein**

The results from biochemical studies of DnaA E204Q protein described above showed that the intrinsic ATPase activity of DnaA E204Q protein decreased without loss of affinity for ATP and ADP. To determine the physiological role of the intrinsic ATPase activity of DnaA protein, we examined the influence on chromosome DNA replication of the dnaA400 mutation encoding the DnaA E204Q protein. We assumed that the ATPase activity is involved in negative regulation of oriC DNA replication. If such is indeed the case, the introduction of the dnaA400 gene into wild-type E.coli cells should lead to an overinitiation of chromosomal DNA replication, in an oriC-dependent manner, resulting in a lethal phenotype of the mutant. The coding regions of the wild-type dnaA gene and the dnaA400 gene were conjugated with the dnaA promoter, and resultant plasmids were introduced into a wild-type strain (JM109). As shown in Table II, the transformation efficiency of pMZ002-1 (dnaA400) was <1/10³ of that of pMZ002-2 (wild-type dnaA). In other words, the dnaA400 gene showed a lethal phenotype in wild-type cells. When the KA450 strain (ΔoriC) was used as a recipient for transformation, the transformation efficiency of pMZ002-1 (dnaA400) was much the same as that of pMZ002-2 (wild-type dnaA) (Table II), indicating that lethality by the dnaA400 gene required functions of the oriC sequence. These results suggest that DnaA E204Q protein does affect initiation of DNA replication from the oriC region, the result being the lethal phenotype. However, the possibility that genotype differences between strains (JM109 and KA450) influence the outcome of results in Table II cannot be ruled out at this stage.

**Overinitiation of DNA replication by induction of DnaA E204Q protein in cells**

We next examined the mechanism of lethality by the dnaA400 mutation. A coding region of the dnaA400 gene was introduced into the 3' downstream region of the lac promoter to construct a system where the expression of DnaA E204Q protein is under the control of the lac promoter (pMZ003-1). JM109 cells harboring pMZ003-1 were labeled continuously with [3H]thymine in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM), and incorporation of thymine into trichloroacetic acid (TCA)-insoluble fractions was measured. The rate of thymine incorporation into JM109 cells harboring pMZ003-1 (dnaA400) was 3-fold more than that into JM109 cells harboring pMZ003-2 (wild-type dnaA) (Figure 6). In other words, induction of DnaA E204Q protein caused an overinitiation of DNA replication in cells. After addition of IPTG, the growth rate of cells harboring pMZ003-1 (dnaA400) was slower than that of cells harboring pMZ003-2 (wild-type dnaA) (data not shown). Therefore, the higher incorporation of [3H]thym-
Discussion

In the present study, we tested our hypothesis that the intrinsic ATPase of DnaA protein negatively regulates the initiation of chromosomal DNA replication in cells. We designed mutant DnaA protein (DnaA E204Q), whose intrinsic ATPase activity is lower than that of wild-type DnaA protein, and observed the influence of expression of the mutant protein on DNA synthesis in cells. Transformation of wild-type cells with a plasmid carrying the mutant dnaA gene, encoding the DnaA E204Q protein, caused a lethal phenotype, and expression of the mutant DnaA protein stimulated DNA synthesis. Therefore, the lethal phenotype is apparently caused by overinitiation of chromosomal DNA replication, as a result of a lack of negative regulation of the initiation of DNA replication by the intrinsic ATPase activity of DnaA protein.

DnaA E204Q did show activity in oriC replication systems; however, the specific activity was lower than that of the wild-type protein. Since DnaA protein bound to non-hydrolyzable analogs of ATP was seen to be active in DNA replication in vitro (Sekimizu et al., 1987), hydrolysis of ATP bound to DnaA protein does not contribute to the replication reaction. Therefore, the lower specific activity of DnaA E204Q protein cannot be caused by the lower ATPase activity. A conformational change of DnaA protein by the mutation (Glu204→Gln) may decrease its activity in DNA replication or render it sensitive to denaturation during the course of purification. The ADP-binding form of DnaA E204Q, like wild-type protein, was inactive in DNA replication. Therefore, the overinitiation of DNA replication induced by expression of DnaA E204Q protein in cells is not due to the fact that the mutant protein is insensitive to inactivation by ADP binding. Thus, the character of the DnaA E204Q protein differs from that of the DnaAcos protein, which is not susceptible to inactivation by ADP binding.

An inactivation factor specific for DnaA protein (IdaA protein) has been identified (Katayama and Crooke, 1995). The inactivation activity of IdaA protein required ATP and DNA (Katayama and Crooke, 1995), which are necessary for the intrinsic ATPase activity of DnaA protein (Sekimizu et al., 1987). Recently, we identified a stimulatory activity for DnaA ATPase in a crude extract from E. coli cells (unpublished results). The stimulatory activity co-migrates with the inactivation activity of DnaA protein, under several chromatographic conditions (unpublished results). These results suggest that IdaA protein inhibits oriC replication in vitro by enhancing the intrinsic ATPase activity of DnaA protein. Together with our present data, we propose that IdaA protein contributes to the negative regulation of initiation of chromosomal DNA replication in vivo by its stimulatory activity for DnaA ATPase.

DnaAcos protein is not inactivated by IdaA protein (Katayama, 1994). The resistance of DnaAcos protein to IdaA protein may be due to the fact that this protein loses its affinity for ATP and ADP (Katayama, 1994) and thus cannot take on the ADP-binding form, which is inactive in oriC replication. DnaAcos protein in the absence of ATP probably take on a conformation similar to the ATP-binding form of wild-type DnaA protein (Katayama et al., 1995).

A common feature seen in DnaA protein and GTP-binding protein (G protein), an important factor for the signal transduction system in eucaryotic cells, is that their activities are regulated by forms of purine nucleotide bound to proteins. GTP bound to G protein is slowly hydrolyzed to GDP by the intrinsic GTPase activity of G protein to form the inactive, GDP-binding form (Gilman, 1987). This reaction corresponds to hydrolysis of the ATP bound to DnaA protein by its intrinsic ATPase activity. The GTPase activity of G protein is stimulated by GTPase-
activating protein (GAP), which negatively regulates signal transduction (McCormick, 1989). We consider that IdA protein of E.coli corresponds to GAP in eucaryotic cells (Katayama and Crooke, 1995). Coupled with binding of the ligand to the receptor, G protein bound to GDP became active, i.e., the GTP-binding form was produced, by the exchange reaction of GDP with GTP. We propose that acidic phospholipids in membranes of E.coli give signals to DnaA protein corresponding to the role of the receptor in eucaryotic signal transduction, because acidic phospholipids, such as cardiolipin and phosphatidylglycerol, stimulate the exchange reaction of GDP bound to DnaA protein with ATP by reducing the affinity of DnaA protein for ATP by reducing the affinity of DnaA protein for ATP. In the presence of ATP, DnaA protein is phosphorylated by cAMPdependent protein kinase (A.K.) and this form is necessary for the initiation of the E.coli chromosome replication (Kirk et al., 1978; Castuma et al., 1993; Mizushima et al., 1996a), and that acidic phospholipids are required for oriC DNA replication in vivo (Xia and Dowhan, 1995).

Materials and methods

Bacterial strains

Strains JM109 (recA1, endA1, gyrA46, thi-1, hsdR17, supE44, relA1, Δlac-proAB)::Tn5 [trpD36, proAB Δlac, lacZΔM15]), BW313 (HfrK, 16PO/45[trsl-61-2]/dad1, ungI, thi-1, relA1) and KA450 (oriC1071::Tn10, rnaI198Am), dnaA17(Am), trpE9829Am, trpD(Tr), thrE(Tr), thrI, thyA were from Katayama (1994) and WM435 (dnaA204, leu19, pro19, trp25, his47, thyA59, arg28, met55, deoB23, lacI, strA56, sul1, hisD82) was from our laboratory stock.

Site-directed mutagenesis of the dnaA gene

Site-specific mutation was performed according to the methods of Kunkel (1987). Phase M13mp19, which contains an antisense strand of the coding region of the dnaA gene at the polylinker site (BamHI–HindIII) (Guo, Miki and Sekimizu, unpublished), was introduced into E.coli BW313 (ungI) and the uracil-containing single-stranded DNA was isolated. An oligonucleotide primer, 5′-ATATGCACTCCCAAGCCG-3′, was used as a mismatch primer for replacement of Glu204 by Gln in GlnA (the changed base is indicated in bold). The complementary DNA strand was synthesized in vitro with the uracil-containing single-stranded DNA and the primer. The resultant double-stranded DNA was introduced into JM109. According to the results of DNA sequence analysis of two of three independent plagues contain the mutation. Double-stranded DNA (pMZ001-1 with the mutation, pMZ002-0 without the mutation) was prepared and used for the construction of plasmids.

Construction of plasmids

We used the plasmid pKA231, which is a derivative of pKA233-5 (Katayama, 1994) and contains the wild-type dnaA gene under the control of the arabinose operon, to construct the plasmid for overproduction of DnaA protein for ADP and ATP-binding assay. In the presence of ATP, DnaA protein is phosphorylated by cAMP-dependent protein kinase (A.K.) and this form is necessary for the initiation of the E.coli chromosome replication (Kirk et al., 1978; Castuma et al., 1993; Mizushima et al., 1996a), and that acidic phospholipids are required for oriC DNA replication in vivo (Xia and Dowhan, 1995).

ATP or ADP-binding assay

The ATP- or ADP-binding activity of DnaA protein was determined by filter-binding assay (Sekimizu et al., 1987). DnaA protein (1 pmol) was incubated with [γ-32P]ATP or [γ-32P]ADP at 0°C for 15 min in 40 μl of buffer G (50 mM HEPES/KOH, pH 8.0, 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM ammonium sulfate, 17% (v/v) glycerol and 0.005% Triton X-100). Samples were passed through nitrocellulose membranes (Millipore HA 0.45 μm). The radioactivity remaining on the filters was counted in a liquid scintillation counter.

Measurement of hydrolysis of ATP bound to DnaA protein

The ATPase activity of DnaA protein was measured by the incorporation and thin layer chromatography. DnaA protein (1 pmol) was incubated with 1.5 μM [γ-32P]ATP (10 c.p.m./pmol) at 0°C for 15 min and further incubated at 38°C in a reaction mixture containing 40 mM HEPES/KOH, pH 7.6, 1.1 mM magnesium acetate, 0.5 μM GTP, CTP and ATP, 2 mM ATP, 100 μM dNTP, 7% polyvinyl alcohol, 40 mM creatine phosphate, 100 μg/ml creatine kinase and 200 ng of M13E10. Anti-DnaA serum and protein A-Sepharose in buffer L (50 mM HEPES/KOH, pH 7.6, 5 mM magnesium acetate, 1 mM EDTA, 5 mM ammonium sulfate, 0.005% Triton X-100, 100 mM NaCl, 0.1 mM ATP, 0.1 mM ADP and 5 mg/ml lysozyme) was added to the mixture, which was then incubated at 4°C for 30 min, with rotation. The mixtures were centrifuged at 4500 rpm for 1 min and washed with buffer L and buffer M (buffer L without lysozyme). Precipitates were extracted with 40 μl of 1 M HCOOH and 5 mM each of ATP, ADP and AMP. Samples (0.5 μl) were spotted on P81-cellulose plates (Merck). Chromatography was performed with 1 M HCOOH, 0.5 M LiCl and the plates were autoradiographed.

oriC DNA replication in vitro in a crude extract

Replication of minichromosomes in a crude extract (fraction II) was assayed as described (Fuller et al., 1981). Template DNA (200 ng, 600 pmol of nucleotides), M13E10, 240 μg of fraction II from WM433 (dnaA204) and DnaA protein were mixed with reaction cocktails (Fuller et al., 1981) and incubated for replication at 30°C for 20 min. The reaction was terminated by chilling on ice and adding 10% TCA. Samples were passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter, and the amount of DNA synthesized (pmol of nucleotides) was calculated.

oriC replication system in vitro reconstituted from purified proteins

Reaction cocktails (Sekimizu et al., 1988a) (25 μl) contained 20 mM Tris–HCl (pH 7.4), 0.1 mg/ml bovine serum albumin, 8 mM DTT, 0.01% Brij 58, 58 mM magnesium acetate, 125 mM potassium glutamate, 2 mM ATP, 0.5 mM each of GTP, CTP and UTP, 200 ng of pBSorico (600 pmol of nucleotide), 400 ng of SSB, 40 μg of β subunit of DNA polymerase III, 10 ng of Hu, 180 ng of GyrA, 360 ng of GyrB, 120 ng of DnaB, 90 ng of DnaC, 10 ng of primase, various amounts of DnaA, 450 ng of DNA polymerase III* and 0.1 mM each of dATP, dGTP, dCTP and [γ-32P]dTTP (50–150 c.p.m./pmol). The reaction was terminated by chilling on ice and adding 10% TCA. Samples were passed through Whatman GF/C glass-fiber filters and the amount of radioactivity on the filter was measured in a liquid scintillation counter, while the amount of DNA synthesized (pmol of nucleotides) was calculated.

Measurement of chromosomal DNA replication in vivo

Cells were grown to full growth in LB medium containing [3H]thymine (Sigma) (25 μg/ml, 55 Ci/mmol) for 12 h. A portion of this culture was chilled on ice and adding 10% TCA. Samples were passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter.
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